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EXHIBIT

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## Histopathology of In-Stent Restenosis in Patients With Peripheral Artery Disease

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**Background** Clinical studies have suggested that smooth muscle cell (SMC) hyperplasia is the most likely cause of in-stent restenosis. However, pathological data regarding this issue are limited. Specifically, direct evidence of proliferative activity in tissues excised from stenotic stents has not been previously reported.

**Methods and Results** Tissue specimens were retrieved by directional atherectomy from 10 patients in whom in-stent restenosis complicated percutaneous revascularization of peripheral artery disease. Analysis of cellular composition was performed quantitatively after cell-specific immunostaining. For specimens preserved in methanol (7 of 10), cellular proliferation was evaluated by use of antibodies to proliferating cell nuclear antigen (PCNA), cyclin E, and cdk2. TUNEL staining for apoptosis was performed on 8 paraformaldehyde-preserved specimens. Each of the 10 specimens contained extensive foci of hypercellularity composed predominantly of SMCs

(mean  $\pm$  SEM,  $59.3 \pm 3.0\%$ ). Evidence of ongoing proliferative activity was documented in all 7 methanol-preserved specimens:  $24.6 \pm 2.3\%$  of SMCs were PCNA-positive,  $24.8 \pm 3.1\%$  were cyclin E-positive, and  $22.5 \pm 2.2\%$  were cdk2-positive. Apoptotic cells were detected in all 8 specimens that had been appropriately preserved to permit DNA nick-end labeling. Macrophages and leukocytes were identified in each of the 10 specimens but accounted for a proportionately smaller number of cells ( $14.5 \pm 1.9\%$  and  $9.5 \pm 1.4\%$ , respectively). Organized thrombus was observed in 6 of the 10 specimens.

**Conclusions** These findings support the notion that in-stent restenosis results from SMC hyperplasia and suggest that adjunctive therapies designed to inhibit SMC proliferation may further enhance the utility of endovascular stents. (*Circulation*. 1997;95:1998-2002.)

**Key Words** • stents • restenosis • muscle, smooth • cyclins

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Percutaneous delivery of balloon-expandable stents has been shown to reduce the frequency of restenosis.<sup>1,2</sup> When restenosis does occur within a stent, such lesions have been considered to result from intimal proliferation.<sup>3</sup> Published pathology data regarding this issue in humans, however, are limited.<sup>4,5</sup> Specifically, direct evidence of proliferative activity in tissue retrieved from stenotic stents has not been reported previously. Because clinical experience has suggested that directional atherectomy for treatment of in-stent restenosis is unsuitable for coronary arteries,<sup>6,9</sup> we studied a consecutive series of 10 specimens retrieved by directional atherectomy from patients with in-stent restenosis of noncoronary sites. Cell type-specific and cyclin-specific immunostains were used to identify evidence of cellular proliferation and thereby characterize the basis for in-stent restenosis.

### Methods

#### Patients

Clinical details regarding the patients from whom the specimens described in this report were retrieved are summarized in Table 1.

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#### Specimen Retrieval and Preparation

All specimens were retrieved percutaneously by directional atherectomy.<sup>10</sup> Tissues were fixed in either 10% buffered formalin ( $n=3$ ) or 100% methanol ( $n=7$ ). In addition, a portion of tissue from 8 patients was also preserved in 4% paraformaldehyde fixative to permit DNA nick-end labeling (see below). All tissues were embedded in paraffin, cut at 5- $\mu$ m intervals, and stained with hematoxylin-eosin, elastic trichrome, and immunohistochemical stains for SMCs, macrophages, and leukocytes (see below). Immunostaining for PCNA, cyclin E, and cdk2 were used to establish evidence of proliferative activity among cellular elements in the atherectomy specimens.<sup>11</sup> Because formalin fixation has previously been shown to attenuate the antigenicity of PCNA,<sup>11-12</sup> we limited immunostaining for PCNA, cyclin E, and cdk2 to sections from those specimens that had been preserved in 100% methanol; sections of human tonsil<sup>12</sup> were used as a positive control.

#### Antibodies

Immunohistochemical staining for proliferating cells was carried out with a mouse MAb against human PCNA (Signet) as well as two rabbit polyclonal antibodies against cyclin E and cdk2 (Santa Cruz). SMCs and macrophages were identified with an  $\alpha$ -actin alkaline phosphatase-conjugated MAb (Sigma) and HAM-56 MAb (Enzo), respectively. Leukocytes were identified with an MAb to CD-45 (Dako).

#### Immunohistochemistry

Immunoperoxidase staining was performed on serial sections.<sup>14</sup> This involved blocking endogenous peroxidase with 3% hydrogen peroxide, preincubation in blocking serum, and applying the primary antibody at the appropriate dilution (PCNA, 1:40; cyclin E, 1:100; cdk2, 1:400;  $\alpha$ -actin, 1:300; HAM-56, undiluted; and CD-45, 1:50) overnight at 4°C. A biotinylated

## Selected Abbreviations and Acronyms

MAb = monoclonal antibody  
PCNA = proliferating cell nuclear antigen  
SMCs = smooth muscle cells

anti-mouse or anti-rabbit secondary antibody (Signet) was then applied for 30 minutes at room temperature, followed by a streptavidin-horseradish peroxidase complex. Sections were rinsed with PBS and visualized by incubation with either 0.05% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride dihydrate, 3-amino-9-ethylcarbazole, or fast red substrate. A counterstain of 10% Gill's hematoxylin was applied before coverslipping. To detect apoptosis *in situ*, fragmented DNA was nick-end labeled with biotinylated dUTP introduced by terminal deoxytransferase and then stained with avidin-conjugated peroxidase.<sup>13</sup> For each tissue, one section was processed as a positive control by pretreatment with DNase I, 1  $\mu$ g/mL (Sigma), and one negative control was incubated in the absence of the terminal deoxytransferase enzyme.

## Light Microscopic Analysis

Hematoxylin-eosin-stained sections were used to assess the presence of inflammatory cell infiltrates, calcific deposits, organized thrombus, foam cells, and cholesterol clefts. Elastic tissue-stained sections were used to assess the presence of media and adventitia. Quantitative analysis for each immunostain was performed by manually counting all cells in five randomly chosen high-power fields ( $\times 600$ ); results of these quantitative analyses are reported as the percentage (mean  $\pm$  SEM) of positively stained cells among the five high-power fields.

## Results

The quantitative results of pathological examinations performed on atherectomy specimens retrieved from sites of in-stent restenosis are summarized in Table 2.

## Cellularity

All 10 specimens contained extensive foci of hypercellularity (Fig 1). These foci were composed of  $\alpha$ -actin-positive cells with phenotypic characteristics of "activated" SMCs,<sup>12</sup> including stellate morphology, surrounded by a loose, light-staining extracellular matrix. In all 10 specimens,  $\alpha$ -actin-positive cells represented the predominant cell type (mean  $\pm$  SEM, 59.3  $\pm$  3.0%).

Evidence of ongoing proliferative activity was documented in all 7 specimens that were preserved in methanol fixative for immunostaining with antibodies to PCNA, cyclin E, and cdk2. Double immunostaining (Fig 2) indicated that most PCNA-, cyclin E-, and cdk2-positive cells were SMCs. Analysis of five randomly selected high-power ( $\times 600$ ) fields from each of these 7 specimens indicated that 24.6  $\pm$  2.3% (16.7% to 36.0%) of the SMCs were PCNA-positive, 24.8  $\pm$  3.1% (14.0% to 40.5%) were cyclin E-positive, and 22.5  $\pm$  2.2% (12.8% to 30.0%) were cdk2-positive.

## Apoptosis

Apoptotic cells were detected in all 8 specimens that had been appropriately preserved to permit DNA nick-end labeling (Fig 2). The extent of apoptotic cells (12.2  $\pm$  2.9%) was much greater than that described previously in atherectomy specimens retrieved from restenosis lesions.<sup>14</sup>

Macrophages and leukocytes were identified in all 10 specimens but accounted for a proportionately smaller number of cells (14.5  $\pm$  1.9% and 9.5  $\pm$  1.4%, respectively) (Fig 2). Foam cells were not observed in any specimen.

## Thrombus

Thrombus was observed in 6 (60.0%) of 10 specimens; in all cases, thrombus was organized and integral to the excised tissue, as opposed to periprocedural collections of red blood cells. In all 10 cases, thrombus was limited to  $<5\%$  of the area of the specimen.

## Other Findings

Media was observed in one specimen retrieved from the superficial femoral artery; this portion of the specimen was excluded from further analysis. Adventitia was not observed in any specimen. Focal calcific deposits were limited to a single specimen.

## Discussion

Because stents are considered to neutralize geometric effects, including remodeling,<sup>16</sup> development of restenosis after stent implantation has been attributed principally to SMC proliferation. Successful application of intravascular radiation to limit thickening of stent neo-

TABLE 1. Clinical Findings in 10 Patients With In-Stent Restenosis

Patient	Sex	Age, y	Type	No.	Stent				Stent-DA Interval, mo
					Site	D <sub>i</sub> , mm	CSA <sub>i</sub> , mm <sup>2</sup>	CSA <sub>r</sub> , mm <sup>2</sup>	
1	F	69	SEx	2	SFA	4.8	17.0	8.6	11
2	F	38	BEEx	1	SFA	5.3	22.2	7.0	19
3	M	84	BEEx	3	SFA	4.7	17.8	5.1	7
4	M	65	BEEx	2	SFA	4.8	18.9	4.2	6
5	F	65	BEEx	4	SFA	4.4	19.6	5.1	6
6	M	65	BEEx	3	SFA	5.7	25.3	7.4	12
7	F	53	SEEx	1	SCA	6.5	25.0	1.9	4
8	F	48	BEEx	1	CIA	7.3	38.5	2.2	16
9	M	64	BEEx	5	LIA	6.5	31.8	2.9	25
10	M	67	SEEx	1	RIA	6.0	28.6	3.8	10

No. indicates number of stents; D<sub>i</sub>, initial diameter; CSA<sub>i</sub>, initial luminal cross-sectional area; CSA<sub>r</sub>, luminal cross-sectional area at time of DA for restenosis; DA, directional atherectomy; SEEx, self-expanding (Wallstent, Schneider); BEEx, balloon-expandable (Palmaz, Johnson & Johnson Interventional Systems); SFA, superficial femoral artery; SCA, subclavian artery; CIA, common iliac artery; LIA, left iliac artery; and RIA, right iliac artery.

<sup>a</sup>In three patients (patients 2, 4, and 5), mild to moderate stenosis extended beyond the stent margins; in the remaining patients, stenosis was limited to the stent margins.

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TABLE 2. Pathological Findings in 10 Patients With In-Stent Restenosis

Patient	Coll Density/hpf	No. (%) $\alpha$ -Actin+/hpf	No. (%) HAM-55+/hpf	No. (%) CD45+/hpf	No. (%) PCNA+/hpf	No. (%) Cyclin E+/hpf	No. (%) cdk2+/hpf	No. (%) TUNEL+/hpf	Thrombus
1	36.0	16.0 (44.4)	7.4 (20.6)	7.0 (19.4)	8.0 (22.2)	9.0 (25.0)	9.6 (26.7)	9.4 (26.1)	+
2	38.4	22.2 (57.8)	3.0 (7.8)	2.2 (5.7)	ND	ND	ND	4.2 (10.9)	+
3	60.6	36.2 (59.7)	10.0 (16.5)	4.8 (7.9)	ND	ND	ND	ND	0
4	51.8	26.2 (50.8)	9.8 (19.0)	5.8 (11.2)	13.4 (26.0)	11.2 (21.7)	9.8 (19.0)	5.0 (9.7)	+
5	52.2	37.8 (72.4)	7.8 (14.6)	5.2 (10.0)	ND	ND	ND	2.4 (4.8)	0
6	51.6	31.2 (60.6)	10 (18.4)	6.4 (12.4)	10.6 (20.5)	12.8 (24.8)	13.2 (25.6)	ND	+
7	49.6	27.2 (54.8)	3.8 (7.7)	3.0 (6.0)	13.4 (27.0)	14.0 (28.2)	11.6 (23.4)	5.8 (11.7)	0
8	40.0	22.4 (56.0)	9.2 (23.0)	3.8 (9.5)	14.4 (36.0)	16.2 (40.5)	12.0 (30.0)	9.4 (23.6)	0
9	52.8	40.4 (76.5)	4 (7.5)	2.4 (4.5)	8.8 (16.7)	7.4 (14.0)	6.8 (12.8)	2.8 (4.9)	+
10	50.8	30.4 (59.8)	4.4 (8.7)	4.0 (7.9)	12.2 (24.0)	10.0 (19.7)	10.0 (19.7)	3.0 (5.9)	+

hpf indicates high-power field; +, positive; and ND, not done.

intima in a variety of animal models,<sup>17-19</sup> on the premise that proliferating cells display enhanced sensitivity to ionizing radiation, constitutes indirect support for this notion. Likewise, accelerated endothelialization of stents after catheter delivery of the gene encoding vascular endothelial growth factor has been shown to markedly reduce SMC proliferation and thereby reduce luminal narrowing due to thickening of the stent neointima.<sup>20</sup>

Histological findings reported for a limited number of patients have suggested that in-stent restenosis is characterized by SMC hyperplasia. Pathological analysis of graft segments surgically retrieved from two patients described by Anderson et al<sup>5</sup> revealed SMCs "with abundant eosinophilic cytoplasm and minimal interstitial tissue" in the tissue overlying the stent wires. Among

four patients with in-stent restenosis described by van Beusekom et al,<sup>4</sup> "... tissue that narrowed the vessels always consisted of SMCs (often with a 'dendritic' appearance) within an extensive extracellular matrix." The extent of residual proliferative activity among these hypercellular foci was not specifically investigated in either series of patients.

The findings in the present series of atherectomy specimens are consistent with the aforementioned histopathology studies. Each specimen contained extensive foci of hypercellularity, predominantly SMCs. Moreover, when these tissues were preserved in fixative specifically intended to preserve the antigenicity of PCNA, immunostaining performed with the corresponding monoclonal antibody disclosed that proliferative activity was abundant in all such specimens. Because

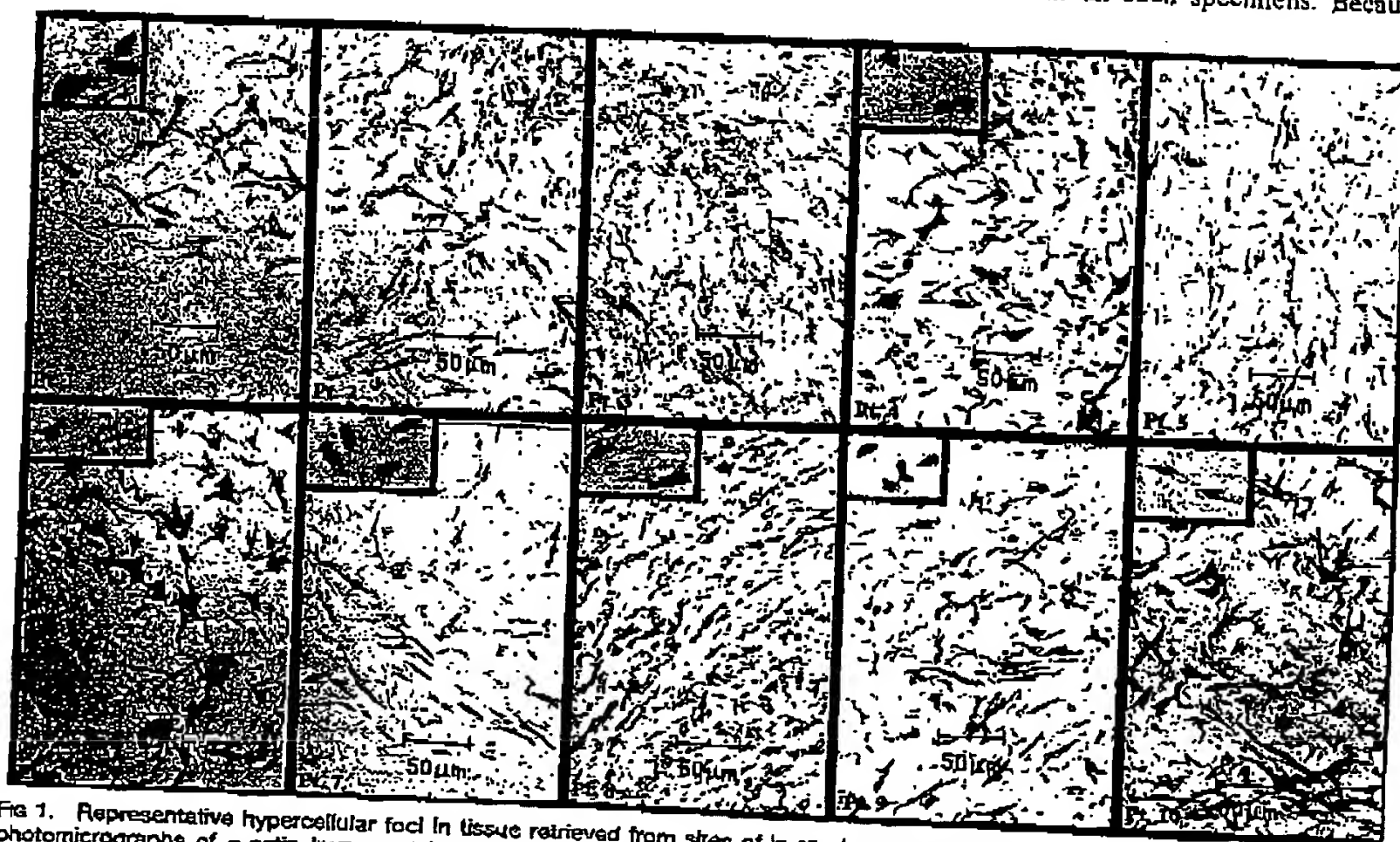


Fig 1. Representative hypercellular foci in tissue retrieved from sites of in-stent restenosis in each of 10 patients (Pt.). Low-power photomicrographs of  $\alpha$ -actin-immunostained sections show abundant cells with phenotypic characteristics of activated SMCs. In methanol-preserved specimens (Pts. 1, 4, 6, 7, 8, 9, and 10), immunostaining for PCNA disclosed evidence of abundant proliferative activity (insets).

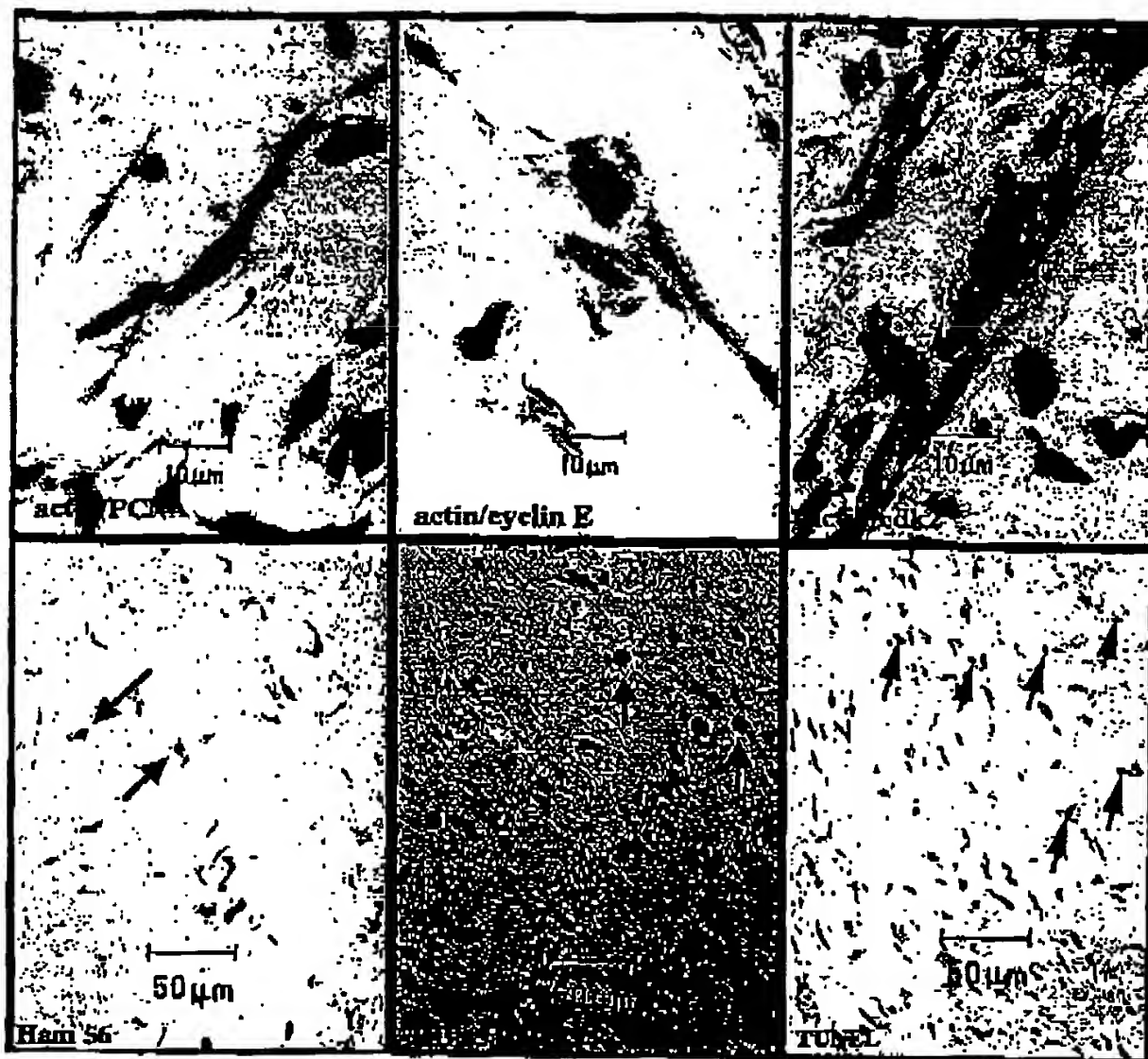


FIG 2. Cell-specific, cell-cycle, and apoptotic immunostaining. Top, Proliferating SMCs double-immunostained for  $\alpha$ -actin and either PCNA, cyclin E, or cdk2. Bottom, Left and center, sparsely distributed macrophages (HAM56) and leukocytes (CD45). Bottom right, TUNEL staining. Arrows identify representative positive staining.

one<sup>21</sup> of three previous studies of native-vessel restenosis lesions<sup>21-23</sup> was interpreted to show a paucity of SMC proliferation after immunostaining for PCNA, we used polyclonal antibodies to two additional cell cycle-regulatory proteins (cyclin 2 and cdk2) to confirm the extent of SMC proliferation observed for in-stent restenosis. Double immunostaining for all three cell cycle proteins and  $\alpha$ -actin confirmed that SMCs accounted for most of the proliferative activity.

The findings in this study constitute the first direct evidence that SMC proliferation contributes to in-stent restenosis in human subjects and implies a potential role for adjunctive therapies intended to reduce in-stent restenosis by inhibiting SMC proliferation.

The extent of cellular proliferation and apoptosis in the present series of in-stent restenosis specimens exceeds that observed in restenosis specimens retrieved from native vessels.<sup>11,14</sup> Cardiovascular and noncardiovascular studies have typically disclosed a relationship between cellular proliferation and apoptosis,<sup>24</sup> a concept that is consistent with the extensive proliferative activity demonstrated in the present series of specimens. The basis for this relationship between proliferative activity and programmed cell death remains to be established, but as suggested previously,<sup>14,23,26</sup> apoptosis may act to limit the extent of cellular accumulation.

Inflammatory cells, including foreign body granuloma formation, have been described in at least one previous animal study.<sup>27</sup> Although neither giant cells nor granulomata were observed in the present series of specimens, occasional inflammatory cells were identified by CD-45 immunostaining. A relatively small HAM-56 macrophage population was identified as well, but the contribution of both cell types to restenosis in these 10 cases appears to be limited.

The role of thrombus in restenosis, including in-stent restenosis, is enigmatic. Schwartz et al<sup>28</sup> suggested that mural thrombus may constitute the primordial infrastructure that is subsequently colonized by activated SMCs. Thrombus was in fact observed in 6 of 10 specimens (60.0%) in the present cohort and thus cannot be excluded as a factor contributing to the genesis of peripheral vascular in-stent restenosis.

The limited number of specimens (10) in the present series may be viewed as a limitation of this study. It should be noted, however, that the opportunity to study a larger number of specimens, particularly coronary specimens, has to date been limited by technical concerns regarding the use of directional atherectomy within stents.<sup>6,9</sup> At least two cases<sup>2,8</sup> of target-stent disruption secondary to (coronary) atherectomy have been reported previously, one of which required urgent bypass surgery. Although animal models have failed to



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disclose evidence of site-specific variation in the histopathology of in-stent neointimal thickening,<sup>429</sup> it must be acknowledged that the extent to which the pathological features of in-stent restenosis described here in the lower-extremity vasculature can be extrapolated to other vascular beds, as well as other types of stents, awaits further confirmation.

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